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Kinetic properties of polyphenoloxidase in organic solvents

A study in Brij 96-cyclohexane reverse micelles

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Polyphenoloxidase entrapped in Brij 96-cyclohexame reverse micelles showed both cresolase activity towards monophenols and catecholase activity towards diphenols. The kinetic parameters, such as apparent K_m, temperature effect and pH profile, were determined for catecholase activity, the findings being very close to those found in aqueous systems. The enzyme activity was dependent on the molecular ratio of water to surfactant (ω_o), obtaining a maximum activity at $\omega_o = 10$. The stability of the system with time increased with the water content until $\omega_o = 10$, and then decreased. An inverse relation was found between activity and water volume fraction (θ) when ω_o was held constant. In this situation V_{max} was constant while K_m varied proportionally with θ.

Polyphenoloxidase; Organic solvent; Reverse micelle; Brij 96

1. INTRODUCTION

Optically transparent, water containing, reverse micelles are capable of solubilizing enzymes in apolar solvents. The new burst of interest in these enzyme-containing micellar systems, as already mentioned in recent review articles [1,2], lies in the biotechnological applications and in the study of fundamental properties of enzymes. Although a number of enzymes have been investigated in such media, most work has been carried out on hydrolases (α-chymotrypsin and lipases) and dehydrogenases but not on oxidases [1]. The surfactants mostly employed are AOT, CTAB, SDS and Triton X-100 [3] but only few enzymes have been entrapped in Brij 96 [4]. We describe here for the first time some kinetic parameters of a membrane bound enzyme such as grape polyphenoloxidase (EC 1.14.18.1) (PPO) in Brij 96/cyclohexane/water reverse micelles.

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Abbreviations: PPO, polyphenoloxidase; AOT, sodium bis-(2-ethylhexyl)sulfosuccinate; CTAB, cetyltrimethylammonium bromide; Brij 96, poly-(10)-oxyethylene oleyl ether; *p*-cresol, 4-methyl phenol; Tris, tris(hydroxymethyl)aminomethane

2. MATERIALS AND METHODS

2.1. *Materials*

PPO was purified as described previously [5]. Brij 96, *p*-cresol, and 4-methyl catechol (Sigma) were used without further purification. The cyclohexane (UV-spectroscopic grade) was from Panreac (Spain). $NaIO_4$ (Merck) was used to mimic the enzyme behaviour to calculate λ_{max} and ϵ of 4-methyl-*o*-benzoquinone, since it is able to oxidize the *o*-diphenol to *o*-quinone [6,7].

2.2. *Preparation of reverse micelles*

Brij 96 microemulsions containing PPO were prepared by injection of microliter volumes of enzyme stock solution (5 mg/ml) with the same volume of 50 mM buffered aqueous solutions into Brij 96/cyclohexane solution (0.55–0.092 M) while being gently shaken for ~1 min at room temperature. The 50 mM buffers used were acetate (pH 4–5.5), phosphate (pH 6–7.5) and Tris (pH 8.0).

2.3. *Enzyme assay*

Kinetic studies were performed on a Uvikon 810 spectrophotometer on-line with an Olivetti M24 personal computer. Catecholase activity was followed by the appearance of 4-methyl-*o*-benzoquinone at 375 nm ($\epsilon = 1591\ M^{-1} \cdot cm^{-1}$) after

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adding PPO entrapped in Brij 96/cyclohexane reverse micelles to a 4-methyl catechol dissolved in cyclohexane. The standard reaction media contained 0.5 mM 4-methyl catechol, 0.166 M Brij 96, $\omega_0 = 10$, $\theta = 0.6\%$, 15 μg/ml of PPO in 50 mM acetate buffer, pH 5.0, at 25°C, unless stated otherwise. Cresolase activity was measured just as catecholase activity except that the reaction mixture contained 10 mM *p*-cresol, 80 μg/ml of PPO in 50 mM phosphate buffer, pH 7.0, $\omega_0 = 10$ and $\theta = 2.4\%$.

3. RESULTS AND DISCUSSION

Using molecular oxygen, PPO entrapped in reverse micelles catalyzes the *o*-hydroxylation of monophenols (like *p*-cresol) to *o*-diphenols (like 4-methyl catechol) (cresolase activity) and the further oxidation of diphenols to *o*-quinones (catecholase activity) as shown in fig.1. The former is characterized by a lag period, defined as the intercept on the abscissa obtained by extrapolation of a linear part of the product accumulation curve. This behaviour is similar to cresolase activity of other tyrosinases or polyphenoloxidases from different sources in aqueous media [8,9].

Dependence of the catecholase activity on pH in reverse micellar solutions is shown in fig.2a. This broad pH optimum between 5.0 and 6.5, with a marked decrease in activity above pH 7.0 and below pH 5.0, is very similar to the pH optimum range in water [5]. However, there is an alkaline shift of one unit of pH with respect to the aqueous solution, which has been described for other

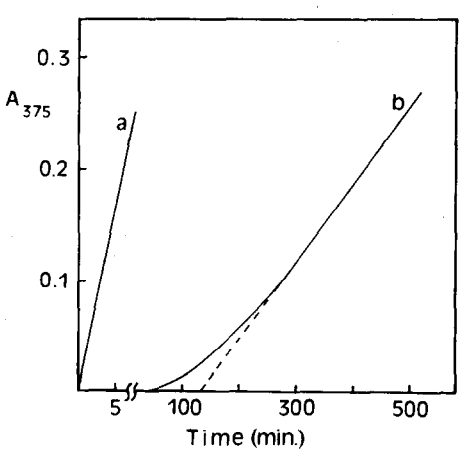

Fig.1. Enzymatic activities of polyphenoloxidase. (a) Catecholase activity. (b) Cresolase activity. Non auto-oxidation of *p*-cresol was detected after 500 min.

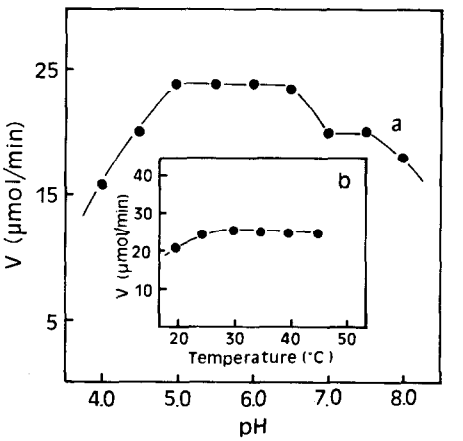

Fig.2. (a) pH profile of catecholase activity. Buffers used were: acetate (pH 4–5.5), phosphate (pH 6–7.5) and Tris (pH 8.0). (b) Effect of temperature on catecholase activity.

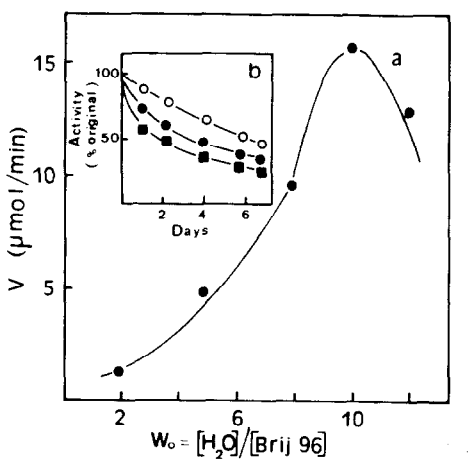

Fig.3. (a) Steady-state rate dependence on ω_0, in a range between 2 and 12 and [Brij 96] between 0.55 and 0.092 M. (b) Enzyme stability of catecholase activity in Brij 96 reverse micelles as function of time. Activity was expressed in % to the initial activity: $\omega_0 = 8$ (●); $\omega_0 = 10$ (○); $\omega_0 = 12$ (■).

reverse micelles formed by other surfactants [10,11].

The effect of temperature on the catecholase activity (fig.2b) shows a broad range of optimum temperature between 25 and 40°C as in water [5]. Above 45°C, the activity decreases to almost zero perhaps due to the modification by the temperature of physical characteristics of the reverse micelles; these changes perhaps include a rearrangement of the surfactant molecules to

create a continuous aqueous phase. The latter phenomenon is known as percolation.

The activity of enzymes in reverse micelles depends on its water content, defined as ω_0 = [H_2O]/[Brij 96]. The bell-shaped profile of the steady-state rate as function of ω_0 (fig.3a) was similar to other enzymes [4,12,13]. The optimum ω_0 was 10. The existence of optimal ω_0 for the reaction rate was probably related to the conformational changes of the protein in the micelle.

The stability of the catecholase activity at different ω_0 is shown in fig.3b. The stability increased with the water content until ω_0 = 10, where its half-life is about one week, and then decreased at greater values of ω_0.

The V_{max} and K_m for 4-methyl catechol was calculated by a double-reciprocal plot at different water contents and compared with 50 mM acetate buffer, pH 5.0, as shown in table 1. The apparent K_m was almost constant (4–5 mM) at ω_0 between 8 and 12, and very close to the 9 mM value obtained in aqueous media [5]. The V_{max} was higher at ω_0 = 10, where the ratio V_{max}/K_m amounted to 21% of that in buffer. These results are similar to the data obtained for other enzymes [3].

Table 1

Michaelis constant and V_{max} of catecholase activity with polyphenoloxidase in 50 mM acetate buffer, pH 5.0, and in reverse micelles at different ω_0

ω_0	K_m[a] (mM)	V_{max} (mM·min^{-1}·mg enzyme^{-1})	V_{max}/K_m (min^{-1}·mg enzyme^{-1})
8	4	5.5	1.37
10	5	12.7	2.54
12	4.5	8.8	1.95
Buffer[b]	9	111.1	12.34

[a] The K_m was expressed with respect to the total volume
[b] Catecholase activity was measured according to [5]

The effect of water volume fraction (θ) in the catecholase activity at ω_0 constant (ω_0 = 10) is shown in fig.4a. When θ was varied from 0.3% to 1% in the reaction media, an inverse dependence was found between activity and % H_2O. For understanding this novel relation between θ and activity, we studied the variation of V_{max} and K_m, finding that the apparent K_m changed proportionally with θ, while V_{max} was constant. This relation seemed to be due to an increase of the interfacial area, which removes substrate from the apolar media to be introduced in the interfacial space of empty reverse micelles, decreasing the availability of substrate from the enzyme. Further support for this conclusion comes from the fact that 4-methyl catechol can act as cosurfactant, forming micelles of ω_0 = 0, which was impossible with the system Brij 96/cyclohexane/water.

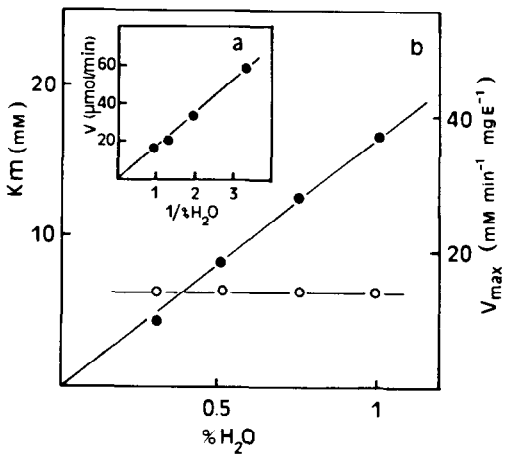

Fig.4. Effect of water volume fraction (θ) at ω_0 = 10 on the catecholase activity at a fixed substrate concentration (0.5 mM) (a) and on the kinetic parameters K_m (●) and V_{max} (○) (b). Water contents were varied between 0.3 and 1% (v/v).

In conclusion, the fact that temperature effect, pH profile and kinetic parameters of PPO in Brij 96 reverse micelles resembled those in the aqueous system, suggested that the catalytic activity was not altered by using this non-ionic surfactant, allowing the study of water-insoluble substrates and the enzyme in similar interfacial conditions in vivo.

Furthermore, the system may have a potential biotechnological interest: in regioselective hydroxylation of monophenols due to the difficulty of synthesis in organic chemistry [14]; in the removal of oily industrial wastewater phenols [15] and in the synthesis of 1,2-benzoquinones for immobilizing enzymes in bioreactors [16].

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